

UNCLASSIFIED

AD NUMBER
ADB265810
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jun 2000. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 21 Feb 2003

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8319

TITLE: Cellular and Molecular Roles of the Akt Protein Kinase in
Breast Carcinomas

PRINCIPAL INVESTIGATOR: Anne Wojtek, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: June 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government
agencies only (proprietary information, Jun 00). Other requests
for this document shall be referred to U.S. Army Medical Research
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland
21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20010507 020

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

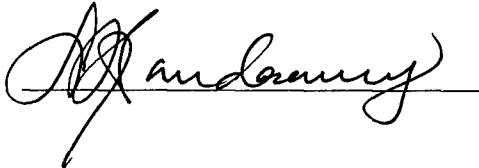
Award Number: DAMD17-98-1-8319

Organization: University of Michigan

Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.



04/21/01

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

June 2000

3. REPORT TYPE AND DATES COVERED

Annual (4 May 99 - 3 May 00)

4. TITLE AND SUBTITLE

Cellular and Molecular Roles of the Akt Protein Kinase in Breast Carcinomas

5. FUNDING NUMBERS

DAMD17-98-1-8319

6. AUTHOR(S)

Anne Vojtek, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)University of Michigan
Ann Arbor, Michigan 48109-0606**8. PERFORMING ORGANIZATION
REPORT NUMBER****E-MAIL:**

avojtek@umich.edu

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, May 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

Akt is a serine/threonine kinase that has been implicated in the initiation and/or progression of breast cancers. In order to gain an understanding of how Akt promotes malignant transformation, we identified proteins that are regulated by phosphorylation by Akt, including a POU domain family transcription factor and the B-Raf serine/threonine kinase. Whereas Akt may upregulate gene expression through phosphorylation of the POU domain transcription factor, Akt negatively regulates B-Raf activity in vitro and in vivo by phosphorylating two residues in the amino-terminal regulatory domain of B-raf. Thus, Akt may promote transformation and cell survival in part by altering gene expression through regulation of transcription factors by phosphorylation and in part by negatively regulating the Ras/Raf pathway by phosphorylating and downregulating the activity of intermediates in this pathway.

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

12

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

☒ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

<u>Anne B. Vojtek</u>	<u>5/30/00</u>
PI - Signature	Date

Table of Contents

Cover.....	
SF 298.....	
Foreword.....	
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	11
References.....	11
Appendices.....	

Introduction

Akt is a serine/threonine kinase that is involved in the initiation and /or progression of breast carcinomas. The cellular and molecular events that are upregulated upon constitutive activation of Akt to promote cancer are not well understood. Akt could induce malignant transformation by phosphorylating key intermediates in a growth signaling pathway. Alternatively, Akt could prevent apoptosis by phosphorylating key components of an apoptosis pathway. The key to understanding how Akt promotes malignant transformation is to identify its cellular targets.

Work Accomplished to Date

For task 1, months 1-18, we proposed to identify effectors and regulators of Akt. We have identified downstream effectors (targets) of Akt, which include Brn1, a POU domain transcription factor, and the B-Raf serine/threonine kinase. We have obtained full length clones of our Akt targets (Task 1, part f), performed in vitro kinase reactions (Task 1, part e), and examined the association between Akt and these targets in vitro and/or in vivo (Task 1, part d). Task one is complete. Experiments directed towards Task 2 are in progress. Our work on the regulation of B-Raf by Akt has been submitted to the *Journal of Biological Chemistry* for review.

POU domain transcription factors may be regulated by Akt

As described in my 1999 DOD Annual Report, we identified a POU domain transcription factor, Brn1, as an Akt interacting protein in a yeast two-hybrid screen and we demonstrated that this protein associates with and is phosphorylated by Akt in vitro. POU domain transcription factors have been reported to promote malignant transformation and cell survival (1, 2). Moreover, four members of the POU domain family, Oct 1, Oct2, Oct3 and Oct11, are expressed in human primary breast carcinomas but not in normal human breast cancer cell lines (3). In addition, the POU domain family protein Brn3b has been reported to strongly repress the promoter of the BRCA-1 tumor suppressor gene in mammary tumor cells (4). Thus, Akt may both promote malignant transformation and induce cell survival through alteration of the regulation of POU family members.

To map the site in Brn1 phosphorylated by Akt, we constructed mutations in the Akt consensus site in Brn1. Akt phosphorylates S or T in an RXXXXS/T motif and Brn1 contains the sequence RKKRTSI, where S is residue 407. We altered the T and S within the Akt consensus site in Brn1 to alanine, each alone and in combination, to create non-phosphorylatable versions of Brn1. The S is the residue that is predicted to be phosphorylated by Akt; the degree to which the adjacent T may be subject to phosphorylation was not known and so this residue was also altered to A. Akt phosphorylates wild type Brn1, the single mutant Brn1 T406A, but not the single mutant Brn1 S407A or the double mutant Brn1 S407A T406A, Figure 1. Thus, Akt phosphorylates Brn1 at residue S407.

POU domain family members contain a conserved Akt consensus site, suggesting that the regulation of POU domain transcription factors by Akt may be a common regulatory mechanism for this family of transcription factors. We have begun to explore the significance of phosphorylation by Akt in two additional family members for which we have obtained full length clones, Brn3a and Brn3c. We will assess the effects of Akt on the ability of these transcription factors to activate a variety of optimized reporters and promoters. We have altered

the S and T within the Akt consensus sites in Brn3c to A to create a non-phosphorylatable version of the transcription factor for these studies.

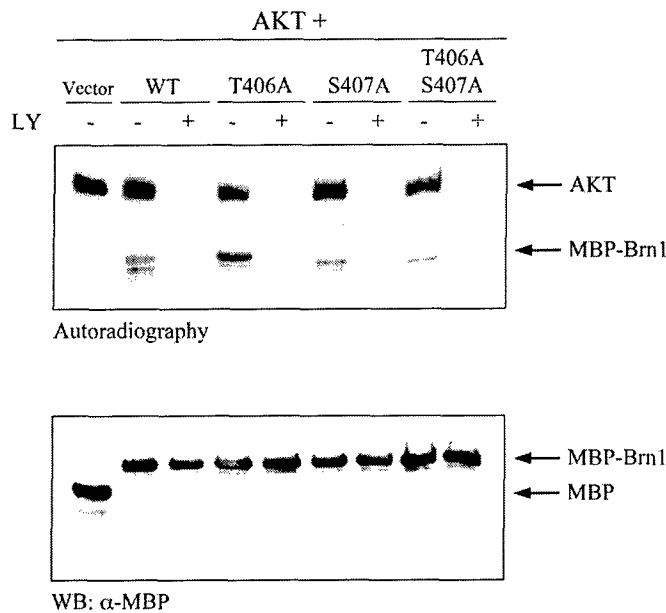


Figure 1. Brn1 is phosphorylated in vitro by Akt at residue S407. MBP-Brn1 fusion proteins, purified from bacteria, were subjected to an in vitro kinase reaction with active Akt (-LY) or inactive Akt (+LY). Akt autophosphorylates and phosphorylates MBP-Brn1 (designated WT, for wild type at the Akt consensus site), but not MBP alone or MBP-Brn1 S407A (upper panel). Western blot of kinase reactions showing equal loading of MBP and MBP-Brn1 fusion proteins (lower panel).

The Ras pathway is negatively regulated by Akt

The Ras signal transduction pathway regulates a number of biological outcomes: cell growth, differentiation, cell survival and cell death. The activation of the Raf serine/threonine kinases by Ras is a key event in each of these processes (reviewed in 5). Coordinated interactions among signaling pathways (cross-talk) plays an important role in cell growth, differentiation, and cell survival and death. We noted that B-Raf contains multiple Akt consensus phosphorylation sites located within its amino terminal regulatory domain. This suggested to us the possibility of cross-talk between the Ras/Raf pathway and Akt. In our studies, we have found that Akt targets and downregulates the activity of the Ras pathway. Akt directly phosphorylates B-Raf. This regulation of B-Raf by Akt negatively regulates its enzymatic activity in vitro and in vivo.

B-Raf contains multiple Akt consensus sites located within its amino terminal regulatory domain. One site, S364, is conserved with c-Raf, but two additional sites, S428 and T439, are unique to B-Raf. We have investigated the role of both the conserved and unique phosphorylation sites in the regulation of B-Raf activity in vitro and in vivo. Phosphorylation of B-Raf by Akt occurs at multiple residues within its amino terminal regulatory domain, at both the conserved and unique phosphorylation sites (S364 as well as S428 and/or T439) (Figure 2). The alteration of the serine residues within the Akt consensus sites to alanines resulted in a progressive increase in B-Raf enzymatic activity in vitro and in vivo (data not shown and Figure 3). Expression of Akt inhibits EGF induced B-Raf activity and inhibition of Akt, with the phosphatidylinositol 3-kinase inhibitor LY294002, upregulates B-Raf activity, suggesting that Akt negatively regulates B-Raf in vivo (Figure 4). In addition, B-Raf and Akt co-associate in vivo (Figure 5).

Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf. This cross talk between the B-Raf and Akt serine/threonine kinases is likely to play an important role in modulating the

signaling specificity of the Ras/Raf pathway and in promoting biological outcome. By understanding the mechanisms by which cells integrate signals, it may be possible to develop strategies to alter cell decisions for the treatment of human diseases:

- to promote cell death under conditions where unrestrained proliferation and cell survival is detrimental (malignant transformation and tumorigenesis); and/or,
- to promote differentiation under conditions where proliferation is detrimental (perhaps promoting differentiation of tumor cells might ameliorate the proliferative/tumorigenic capacity of certain types of tumor cells).

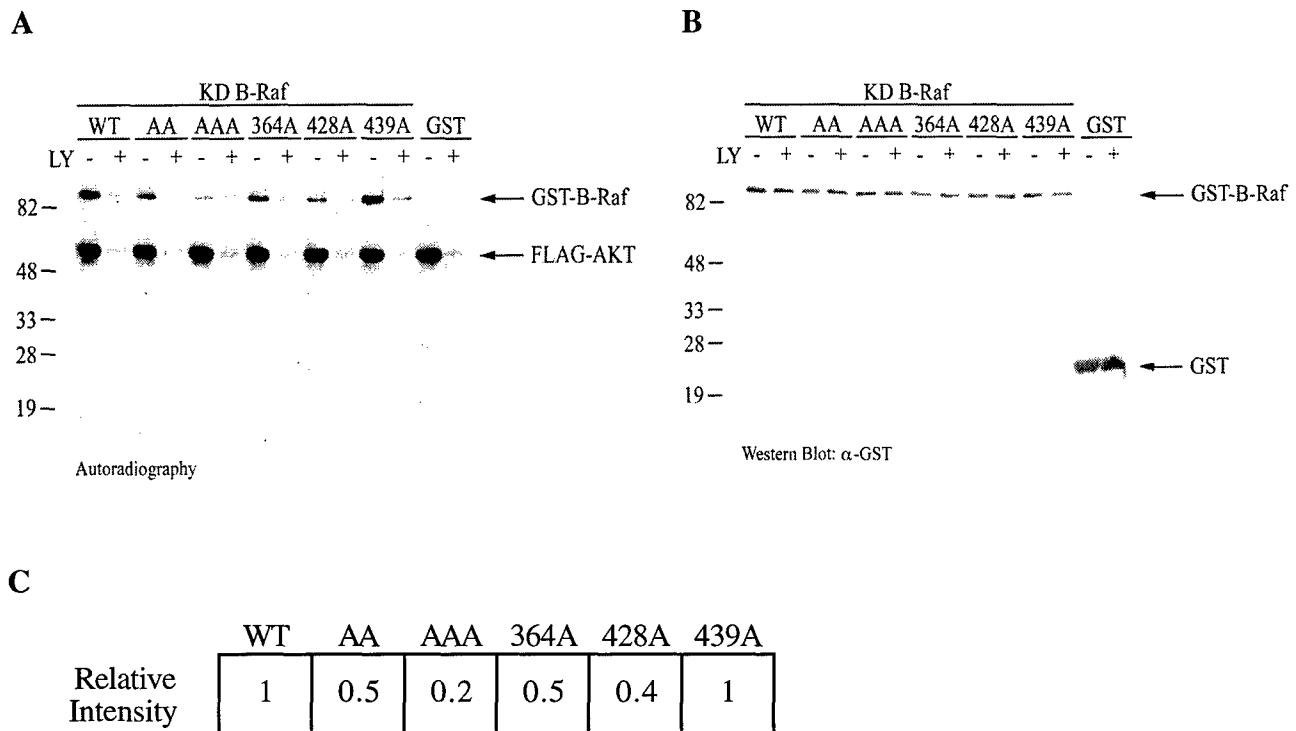
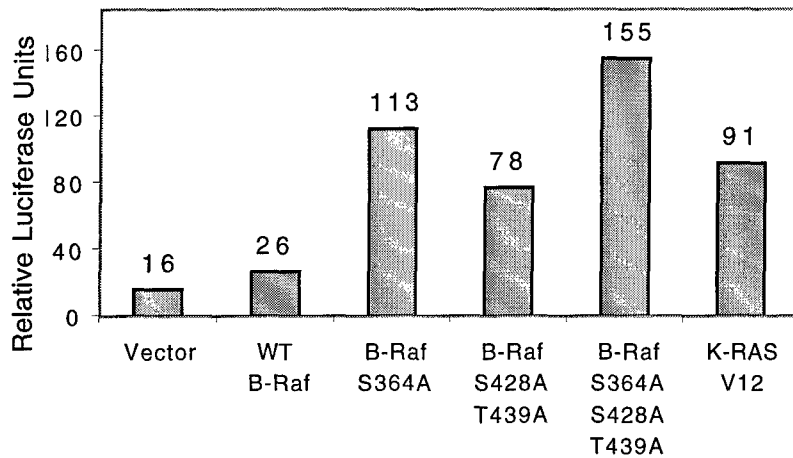


Figure 2. B-Raf is phosphorylated in vitro by Akt at residues 364 and 428. Kinase inactive (K/D) GST-B-Raf fusion proteins were subjected to an in vitro kinase reaction with active Akt (-LY) or inactive Akt (+LY). In addition to the K482M mutation to create a kinase inactive B-Raf, the B-Raf proteins either do not contain additional mutations and are designated WT, for wild type at the Akt consensus sites, or contain mutations at the Akt consensus sites alone (S364A, S428A, or T439A) or in combination (AA: S428A T439A; AAA: S364A S428A T439A). **A.** Autoradiography of the in vitro kinase reactions after SDS-PAGE and transfer to an immobilon filter. Arrows show the position of the phosphorylated K/D GST-B-Raf and the autophosphorylated Flag-Akt. **B.** Western blot analysis of the filter in (A), using an anti-GST antibody, showing equal loading of the GST-B-Raf substrates. **C.** Relative intensity of the phosphorimage signal (-LY lanes) of wild type and mutant K/D GST-B-Raf from the filter in (A).

A



B

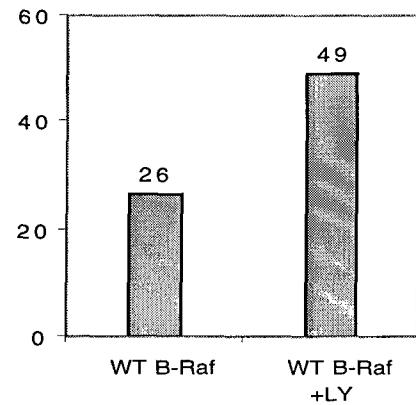


Figure 3. Activation of Elk mediated transcription by B-Raf mutants. **A.** HEK293 cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase and either pcDNA3 (vector control), K-RasV12 (positive control), WT-B-Raf, B-Raf S364A, B-Raf S428A T439A, or B-Raf S364A S428A T439A. **B.** LY294002 (LY) was added for 24 hours prior to harvest, WT-B-Raf+LY. Luciferase activity was assayed 36 hours after transfection. Luciferase activity was normalized to a co-transfected β -galactosidase expression vector. Shown is the average of two experiments performed in duplicate. Western blot of extracts showed that expression of HA-B-Raf wild type and mutants were expressed to comparable levels (data not shown).

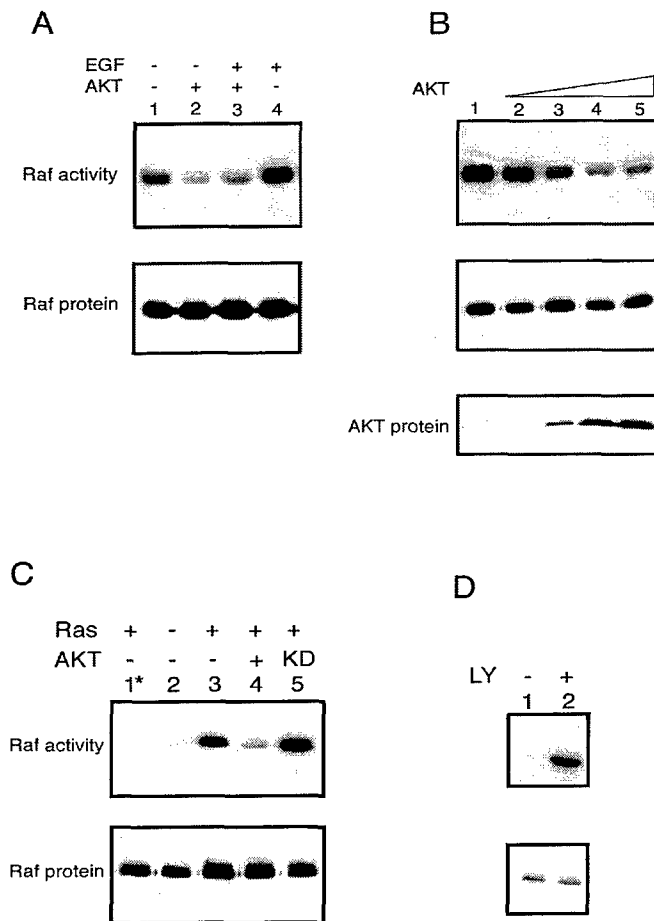


Figure 4. Akt inhibits B-Raf activity in vivo. **A.** HEK293 cells were transfected with expression vectors for HA-B-Raf and Akt, as indicated. B-Raf was immunoprecipitated from lysates and Raf activity assessed by an in vitro coupled kinase reaction in which bacterially expressed GST-Elk-1 was used as substrate (top panel). Lanes 1 and 2: B-Raf was immunoprecipitated from cells 24 hours after transfection. Lanes 3 and 4: B-Raf was immunoprecipitated from cells after serum starvation and stimulation with EGF for 3 minutes. Western blot of HA-B-Raf proteins used for the in vitro kinase reactions (bottom panel). **B.** Akt inhibits Raf enzymatic activity in a dose dependent manner. HEK 293 cells were transfected with the expression vector for HA-B-Raf and increasing concentrations of Akt, as indicated. B-Raf activity was determined by a coupled in vitro kinase reaction (top panel).

(Figure 4 continued) Western blot of HA-B-Raf proteins used in the in vitro kinase reactions (middle panel). Western blot of HA-Akt in cell lysates (bottom panel). **C.** Akt kinase activity is required to inhibit B-Raf activity. HA-B-Raf was transfected with expression vectors for H-RasV12 or Akt as indicated. Activated Ras stimulates the co-transfected Raf activity (compare lanes 2 and 3). GST-MEK was omitted in lane 1 as a control for the specificity of the in vitro Raf kinase assays. The Ras induced Raf activation is inhibited by wild type (lane 4) but not the kinase inactive (lane 5) Akt. **D.** B-Raf activity is enhanced upon inhibition of PI3K. Ha-B-Raf transfected cells were treated with LY294002 for one hour. HA-B-Raf was isolated and kinase activity was determined.

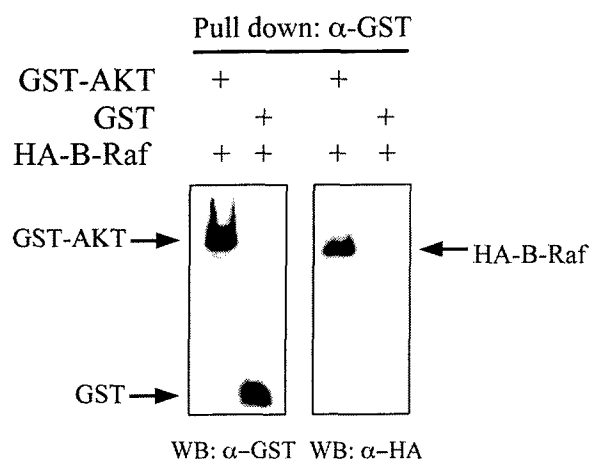


Figure 5. B-Raf and Akt co-associate in vivo. GST-Akt was purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione sepharose. The pull-downs were subject to SDS-PAGE followed by western blotting with antibodies directed against the epitope tags on B-Raf and Akt.

Future Directions

In the coming year, we will continue to focus on the POU domain transcription factors and utilize the S to A and S to D mutants at the Akt phosphorylation consensus sites to define the role that phosphorylation plays in the regulation of the transcriptional activity of these factors. In addition, Akt appears to negatively regulate the Ras pathway at a second step and experiments over the next year will be directed at deciphering the additional actions of Akt on the Ras pathway. We have defined multiple targets of Akt and we will determine the contributions of each of these effector pathways to cell survival and malignant transformation (Task 2).

Key Research Accomplishments

1. Demonstrated that S407 in the Brn1 transcription factor is phosphorylated by Akt
2. Demonstrated that B-Raf is phosphorylated by Akt at multiple residues within its amino terminal domain (S364 and S428)
3. Demonstrated that alteration of the serine residues within the Akt consensus sites in B-Raf to alanine results in a progressive increase in enzymatic activity in vitro and in vivo
4. Expression of Akt inhibits EGF induced B-Raf activity and inhibition of Akt with LY294002 upregulates B-Raf activity, suggesting that Akt negatively regulates B-Raf in vivo
5. Demonstrated that Akt and B-Raf co-associate in vivo
6. Taken together, our results show that the B-Raf serine/threonine kinase is negatively regulated by Akt in vivo and in vitro

Reportable Outcomes

1. Manuscript entitled "Negative Regulation of the serine/threonine kinase B-Raf by Akt" prepared and submitted
2. Grant applications based on work supported by this award prepared and submitted to NIH and ACS
3. This award supports the stipend and tuition of Claudia Figueroa, a graduate student in the Department of Biological Chemistry

Conclusions

Akt is involved in the initiation and/or progression of breast cancers. The cellular and molecular events targeted by Akt to promote cancer are not well documented. In order to gain an understanding of how Akt promotes malignant transformation, we have identified likely targets for Akt. We have found that Akt phosphorylates and likely regulates the transcriptional activity of POU domain transcription factors and that Akt phosphorylates and negatively regulates the Ras/Raf/ERK pathway through phosphorylating the B-Raf serine/threonine kinase. These phosphorylation events, singly and in combination, are likely to be critical elements through which Akt operates to promote cell survival and transformation. Future studies will be directed at determining the contributions of each of these Akt effector pathways in promoting cell survival and in inducing malignant transformation of breast cancer cells (Task 2).

References

1. Smith, M. D., Ensor, E. A., Coffin, R. S., Boxer, L.M. and Latchman, D.S. (1998). Bcl-2 transcription from the proximal p2 promoter is activated in neuronal cells by the Brn-3a POU family transcription factor. *J. Biol. Chem.* **273**, 16715-22.
2. Theil, T., McLean-Hunter, S., Zornig, M. and Moroy, T. (1993). Mouse Brn-3 family of POU transcription factors: a new amino-terminal domain is crucial for the oncogenic activity of Brn3a. *NAR* **21**, 5921-29.
3. Jin, T., Branch, D. R., Zhang, X., Qi, S., Youngson, B. and Goss, P.E. (1999). Examination of POU homeobox gene expression in human breast cancer cells. *Int. J. Cancer* **81**, 104-12.
4. Budhram-Mahadeo, V., Ndsiang, D., Ward, T., Weber, B.L. and Latchman, D. D. (1999). The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells. *Oncogene* **18**, 6684-91.
5. Vojtek, A. B. and Der, C. J. (1999). Complexities of the Ras signaling pathway. *J. Biol. Chem.* **273**, 19925-28.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

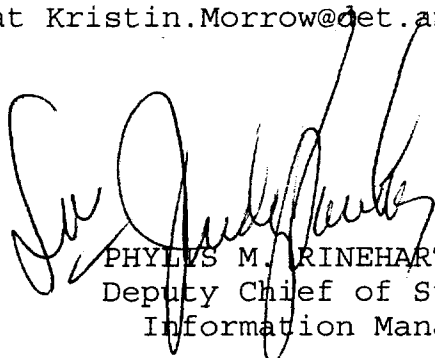
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB263458	ADB282838
ADB282174	ADB233092
ADB270704	ADB263929
ADB282196	ADB282182
ADB264903	ADB257136
ADB268484	ADB282227
ADB282253	ADB282177
ADB282115	ADB263548
ADB263413	ADB246535
ADB269109	ADB282826
ADB282106	ADB282127
ADB262514	ADB271165
ADB282264	ADB282112
ADB256789	ADB255775
ADB251569	ADB265599
ADB258878	ADB282098
ADB282275	ADB232738
ADB270822	ADB243196
ADB282207	ADB257445
ADB257105	ADB267547
ADB281673	ADB277556
ADB254429	ADB239320
ADB282110	ADB253648
ADB262549	ADB282171
ADB268358	ADB233883
ADB257359	ADB257696
ADB265810	ADB232089
ADB282111	ADB240398
ADB273020	ADB261087
ADB282185	ADB249593
ADB266340	ADB264542
ADB262490	ADB282216
ADB266385	ADB261617
ADB282181	ADB269116
ADB262451	
ADB266306	
ADB260298	
ADB269253	
ADB282119	
ADB261755	
ADB257398	
ADB267683	
ADB282231	
ADB234475	
ADB247704	
ADB258112	
ADB267627	